Pseudomonas fluorescens WCS374r-Induced Systemic Resistance in Rice against *Magnaporthe oryzae* Is Based on Pseudobactin-Mediated Priming for a Salicylic Acid-Repressible Multifaceted Defense Response[1][C][OA]

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Selected strains of nonpathogenic rhizobacteria can reduce disease in foliar tissues through the induction of a defense state known as induced systemic resistance (ISR). Compared with the large body of information on ISR in dicotyledonous plants, little is known about the mechanisms underlying rhizobacteria-induced resistance in cereal crops. Here, we demonstrate the ability of *Pseudomonas fluorescens* WCS374r to trigger ISR in rice (*Oryza sativa*) against the leaf blast pathogen *Magnaporthe oryzae*. Using salicylic acid (SA)-nonaccumulating NahG rice, an ethylene-insensitive *OsEIN2* antisense line, and the jasmonate-deficient mutant *hebiba*, we show that this WCS374r-induced resistance is regulated by an SA-independent but jasmonic acid/ethylene-modulated signal transduction pathway. Bacterial mutant analysis uncovered a pseudobactin-type siderophore as the crucial determinant responsible for ISR elicitation. Root application of WCS374r-derived pseudobactin (Psb374) primed naive leaves for accelerated expression of a pronounced multifaceted defense response, consisting of rapid recruitment of phenolic compounds at sites of pathogen entry, concerted expression of a diverse set of structural defenses, and a timely hyperinduction of hydrogen peroxide formation putatively driving cell wall fortification. Exogenous SA application alleviated this Psb374-modulated defense priming, while Psb374 pretreatment antagonized infection-induced transcription of SA-responsive *PR* genes, suggesting that the Psb374- and SA-modulated signaling pathways are mutually antagonistic. Interestingly, in sharp contrast to WCS374r-mediated ISR, chemical induction of blast resistance by the SA analog benzothiadiazole was independent of jasmonic acid/ethylene signaling and involved the potentiation of SA-responsive gene expression. Together, these results offer novel insights into the signaling circuitry governing induced resistance against *M. oryzae* and suggest that rice is endowed with multiple blast-effective resistance pathways.

Plants have evolved a battery of sophisticated defense mechanisms to defend themselves against microbial pathogens. Apart from preformed physical and chemical barriers, plants possess an elaborate matrix of inducible defenses that become activated upon pathogen infection. These inducible responses are regulated by a network of interconnecting signal transduction pathways in which the plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid play central roles (Adie et al., 2007; Robert-Seilaniantz et al., 2007; Asselbergh et al., 2008). A growing body of evidence supports the notion that these signaling pathways do not function independently but influence each other through a complex network of synergistic and antagonistic interactions (Koornneef and Pieterse, 2008). Such cross talk between defense pathways is thought to provide the plant with a cost-efficient regulatory potential to adaptively tailor its defense reaction to the type of attacker encountered.

Besides basal resistance responses that act at the site of pathogen infection, plants are also capable of developing a nonspecific systemic resistance that is effective against future pathogen attack. This phenomenon is known as induced resistance and can be triggered by a variety of biotic and abiotic stimuli (Bostock, 2005). Over the past decade, it has become increasingly clear that the enhanced defensive capacity of induced plants does not necessarily require a direct activation of defenses but can also result from a

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faster and stronger expression of basal defense responses upon pathogen attack. By analogy with a phenotypically similar phenomenon in animals and humans, this enhanced capacity to express infection-induced basal defenses is called "sensitization," "priming," or "potentiation" (Conrath et al., 2002, 2006). In some elegant work on the costs and benefits of priming in Arabidopsis (*Arabidopsis thaliana*), van Hulten and associates (2006) demonstrated that the fitness costs of priming are substantially lower than those of constitutively activated defense. In addition, it was shown that the benefits of priming-mediated resistance outweigh its costs when disease occurs. Priming thus offers an elegant solution to the plant's trade-off dilemma between disease protection and the costs involved in defense activation (Conrath et al., 2006).

The classic example of an inducible plant defense response is systemic acquired resistance (SAR). SAR is triggered by a localized infection with necrotizing microbes and is manifested throughout the plant upon secondary challenge by otherwise virulent microbes (Grant and Lamb, 2006). The onset of SAR is marked by local and systemic increases in endogenously synthesized SA and is tightly associated with the transcriptional reprogramming of a battery of defense-related genes, including those encoding pathogenesis-related (PR) proteins (Ryals et al., 1996; Maleck et al., 2000; Wang et al., 2006). These PR proteins, of which some possess antimicrobial activity, serve as hallmarks of SAR in several plant species and are thought to contribute to the state of resistance attained (Van Loon et al., 2006). Transduction of the SA signal requires the function of NPR1 (also known as NIM1), a master regulatory protein that was identified in Arabidopsis through genetic screens for SAR-compromised mutants (Cao et al., 1994; Shah et al., 1997). Although SA is central to the induction and expression of SAR, it is not the long-distance SAR signal. Instead, exciting new data implicate methyl salicylate and a lipid-derived molecule, possibly JA, as mobile signals for SAR in tobacco (*Nicotiana tabacum*) and Arabidopsis, respectively (Park et al., 2007; Truman et al., 2007).

Colonization of roots by selected strains of nonpathogenic plant growth-promoting rhizobacteria leads to a phenotypically similar form of induced resistance commonly referred to as induced systemic resistance (ISR; Van Loon et al., 1998). Although some rhizobacteria are able to trigger the SA-dependent SAR pathway (De Meyer et al., 1999; Ryu et al., 2003; Tjamos et al., 2005), rhizobacteria-mediated ISR predominantly involves SA-independent signaling (Pieterse et al., 1996; Ahn et al., 2007; Tran et al., 2007). As for the reference strain *Pseudomonas fluorescens* WCS417r, analysis of several well-characterized Arabidopsis mutants revealed that ISR requires an intact response to the plant hormones JA and ET and, like SAR, depends on a functional NPR1 protein (Pieterse et al., 1998; Van Wees et al., 2000). However, downstream of NPR1, the ISR and SAR signaling pathways diverge because, unlike SAR, ISR is not accompanied by the concomitant activation of *PR* genes (Pieterse et al., 1996; VanWees et al., 1997, 1999). Instead, ISR-expressing plants are primed for enhanced expression of predominantly JA- and ET-regulated genes upon pathogen infection (Verhagen et al., 2004; Cartieaux et al., 2008).

Successful establishment of ISR depends on the recognition of bacterial elicitors by the plant roots. Over the past decade, myriad bacterial traits operative in triggering ISR have been identified. Examples include flagella, cell envelope components such as lipopolysaccharides, and secreted metabolites, including antibiotics, quorum-sensing molecules, cyclic lipopeptides, volatiles, and siderophores (Bakker et al., 2007; Ongena et al., 2007; Tran et al., 2007). However, despite the increasing amount of research devoted to the identification and characterization of bacteria-derived ISR elicitors, much remains to be discovered about how these determinants are perceived and ultimately give rise to ISR.

Compared with the vast body of information available for dicotyledonous plants, our understanding of the molecular machinery governing induced resistance responses in monocotyledonous crops is still in its infancy. Evidence demonstrating that central components of the SAR pathway, such as NPR1, are conserved in cereals has only recently been presented (Chern et al., 2001, 2005; Shimono et al., 2007; Yuan et al., 2007). Moreover, reports on SAR- or ISR-like phenomena in monocots are scarce (Kogel and Langen, 2005). Most tellingly in this regard, a 17-year-old report of systemic resistance in rice (*Oryza sativa*) triggered by preinoculation with an hypersensitive response (HR)-eliciting, nonpathogenic *Pseudomonas syringae* strain remains one of the most compelling examples of a monocot SAR-like response to date (Smith and Metraux, 1991). Previously, we reported that root colonization of rice by *Pseudomonas aeruginosa* 7NSK2 renders foliar tissues more resistant to infection by *Magnaporthe oryzae* (De Vleesschauwer et al., 2006). Extensive bacterial mutant analysis and cytomolecular characterization of the defense responses activated in planta revealed that this 7NSK2-mediated ISR acts through secretion of the redox-active pigment pyocyanin, thereby priming systemic tissues for boosted expression of HR-like cell death upon pathogen infection.

Aiming to further dissect the induced systemic resistance response in rice, we analyzed the bacterial determinants and host defense mechanisms underpinning ISR induced by *Pseudomonas fluorescens* WCS374r. This gram-negative bacterium, originally isolated from the rhizosphere of potato (*Solanum tuberosum*), has previously been shown to suppress *Fusarium* wilt (*Fusarium oxysporum* f. sp. *raphani*) of radish (*Raphanus sativus*) and to reduce disease caused by *Ralstonia solanacearum* in *Eucalyptus* (Leeman et al., 1995; Ran et al., 2005a). Remarkably, high inoculum densities of WCS374r cultivated at 28°C failed to elicit ISR in

Arabidopsis against *P. syringae* pv *tomato* (Van Wees et al., 1997), whereas low inoculum densities or inoculum cultivated at elevated temperatures induced resistance against a broad spectrum of pathogens with different parasitic habits (Ran et al., 2005b; Djavaheri, 2007). This wide range of effectiveness of WCS374r-elicited ISR (WCS374r-ISR) strongly suggests that multiple resistance responses are involved. Indeed, recent studies by Ran et al. (2005b) and Djavaheri (2007) demonstrated that WCS374r-ISR against *Turnip crinkle virus* was still functional in Arabidopsis genotypes impaired in JA- and ET-dependent signaling, whereas WCS374r-ISR against *P. syringae* pv *tomato* was blocked in the latter genotypes. Hence, perception of WCS374r seems to result in the activation of multiple signal transduction pathways that all add to establishing broad-spectrum WCS374r-ISR.

In this study, we demonstrate the ability of WCS374r to mount ISR in rice against the leaf blast pathogen *M. oryzae* and provide evidence that this WCS374r-mediated ISR is based on pseudobactin-mediated priming for a pronounced multifaceted cellular defense response. Furthermore, we show that WCS374r-triggered ISR functions independently of SA accumulation but, unlike benzothiadiazole (BTH)-inducible resistance, requires intact responsiveness to ET as well as a functional octadecanoid pathway.

RESULTS

P. fluorescens WCS374r Mounts ISR in Rice to *M. oryzae*

The filamentous ascomycete *M. oryzae* is the causal agent of rice blast disease, one of the most devastating of all cereal diseases and a significant threat to food security worldwide (Talbot, 2003). To determine whether *P. fluorescens* WCS374r-mediated ISR is effective against *M. oryzae*, susceptible rice plants were grown in soil containing WCS374r bacteria and subsequently challenged with the latter pathogen. As a positive control, a subset of the plants was treated with BTH, a functional SA analog and one of the most extensively studied plant defense activators in rice (Nakashita et al., 2003; Ahn et al., 2005; Shimono et al., 2007). Within 4 to 5 d after inoculation, leaves of noninduced control plants developed large, spindle-shaped lesions with a gray center (diameter $>$ 3 mm), often surrounded by chlorotic or necrotic tissue (Fig. 1). In contrast, plants colonized by WCS374r exhibited a marked reduction in the number of these susceptible-type lesions, producing a resistance phenotype characterized by the appearance of many small ($<$1 mm), dark-brown necrotic spots 2 to 3 d after inoculation (Fig. 1). Pooled over four independent experiments, WCS374r pretreatment caused a 47% reduction in lesion number. Application of BTH (0.05 mM) induced an even higher level of protection, reducing the number of susceptible-type lesions by as much as 68% compared with noninduced controls (Fig. 1).

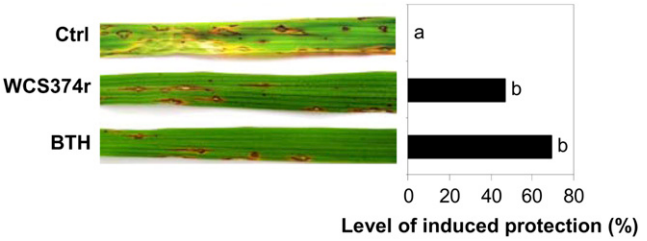

Figure 1. Quantification of ISR and BTH-acquired resistance against *M. oryzae*. ISR was induced by growing rice CO39 plants in soil containing *P. fluorescens* WCS374r bacteria. For chemical induction of blast resistance, plants were soil drenched with BTH (0.05 mM) at 3 d prior to challenge. Control (Ctrl) plants were treated with water. Plants were challenged when 4 weeks old (five-leaf stage) by spraying a spore suspension of virulent *M. oryzae* VT5M1 at 1×10^4 conidia mL^{-1}. Six days after challenge inoculation, disease was rated by counting the number of susceptible-type lesions on the fourth leaves of individual plants and expressed relative to challenged controls. Statistical analysis was performed on data pooled from four independent experiments, since interaction between treatment and experiment was not significant at $\alpha = 0.05$ by analysis of variance. Different letters indicate statistically significant differences between treatments as analyzed by nonparametric Kruskal-Wallis and Mann-Whitney comparison tests ($\alpha = 0.05$, $n > 86$). Photographs depicting representative symptoms were taken at 7 d after inoculation. [See online article for color version of this figure.]

To rule out the possibility that the observed disease protection was due to direct effects of WCS374r on *M. oryzae*, possible spreading of root-inoculated bacteria to foliar tissues was assessed by plating leaf extracts from induced plants onto selective King's medium B (KB) agar plates (King et al., 1954). However, WCS374r bacteria were never detected in leaf blades or sheaths of root-treated plants, indicating that bacterial colonization remained confined to the root zone (data not shown). In conjunction with the inability of WCS374r to inhibit growth of *M. oryzae* in dual-culture experiments (data not shown), these findings strongly suggest that the WCS374r-provoked disease suppression is not due to microbial antagonism but rather results from activation of the plant's own defensive repertoire.

WCS374r-Triggered ISR to *M. oryzae* Is Independent of SA Accumulation But Requires Intact Responsiveness to ET as Well as a Functional Octadecanoid Pathway

To unravel the signaling circuitry governing WCS374r-mediated ISR to *M. oryzae*, bioassays were performed with transgenic and mutant rice lines impaired in various structural components of known defense pathways. As shown in Figure 2, SA-deficient NahG plants (Yang et al., 2004) and the corresponding wild-type line Nipponbare were equally responsive to WCS374r-mediated ISR, suggesting that WCS374r elicits ISR in rice either by activating the SA pathway downstream of SA or by functioning independently of SA. NahG plants also developed wild-type levels of protection against *M. oryzae* in response to treatment

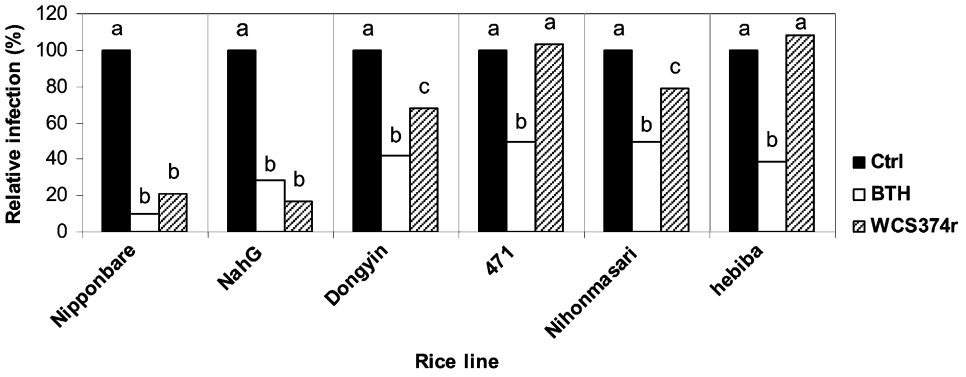

Figure 2. Influence of root treatment with *P. fluorescens* WCS374r or soil drench with BTH on rice blast (*M. oryzae*) severity on different signaling mutants and transgenic rice lines. ISR was induced by growing plants in soil containing WCS374r bacteria. For chemical induction of blast resistance, plants were soil drenched with BTH (0.05 mM) 3 d prior to challenge. Control (Ctrl) plants were treated with water. For details on *M. oryzae* bioassays, see legend to Figure 1. The average numbers of susceptible-type blast lesions on the fourth leaves of individual control plants were 60 (Nipponbare), 56 (NahG), 48 (Dongyin), 59 (471), 65 (Nihonmasari), and 85 (*hebiba*). Within each frame, different letters indicate statistically significant differences between treatments (Kruskal-Wallis and Mann-Whitney; $\alpha = 0.05$, $n > 23$). Data presented are from a representative experiment that was repeated twice with similar results. NahG is a SA-deficient transgenic line generated in the background of Nipponbare; 471 is an ET-insensitive *OsEIN2* antisense line of Dongyin; and *hebiba* is a jasmonate-deficient mutant of Nihonmasari.

with BTH, indicating that SA accumulation is not a prerequisite for the expression of BTH-inducible blast resistance. To investigate whether JA and/or ET play a role in WCS374r-mediated ISR, we tested the effectiveness of WCS374r in the ET-insensitive *OsEIN2*-suppressed transgenic line 471 (Jun et al., 2004) and the JA-deficient mutant *hebiba*, which is impaired in an as yet unidentified step of the octadecadoid pathway (Riemann et al., 2003; Sineshchekov et al., 2004). In contrast to the respective wild-type lines Dongyin and Nihonmasari, both 471 and *hebiba* were blocked in their ability to develop WCS374r-mediated ISR, whereas chemical induction of blast resistance by BTH resulted in levels of induced resistance comparable to those observed in the wild type. The impaired ISR response of 471 and *hebiba* was not due to insufficient root colonization, since WCS374r colonized the rhizosphere of the different rice genotypes to comparable levels (5.4 ± 0.7 log colony-forming units [cfu] g^{-1}). Together, these results suggest that WCS374r-mediated ISR against *M. oryzae* is independent of SA accumulation but, unlike BTH-inducible blast resistance, requires the operation of an ET/JA-regulated signaling pathway.

Involvement of Iron-Regulated Metabolites in the Elicitation of ISR by WCS374r

Several lines of evidence corroborate a major role for iron-regulated bacterial metabolites in WCS374r-mediated ISR in dicotyledonous plants (Leeman et al., 1996; Ran et al., 2005a). To address whether WCS374r mounts ISR to *M. oryzae* in a similar manner, we first compared the ISR-triggering capacity of inoculum cultivated on iron-rich Luria-Bertani (LB) medium to that of inoculum prepared from iron-limited KB. Figure 3A shows that, in contrast to WCS374r prepared from KB, LB-grown bacteria failed to significantly reduce disease severity. Because LB- and KB-grown bacteria colonized rice to a similar extent (data not shown), the observed difference in ISR is likely due to the different iron nutritional state of both inocula. At inoculation, LB-grown inoculum had an internal iron pool visible in the red color of the bacterial pellet, whereas an internal iron pool was not observed for KB-grown WCS374r (data not shown). Although it cannot be excluded that differences in medium composition other than iron content might have contributed to the impaired ISR-triggering capacity of LB-derived inoculum, these observations strongly suggest the involvement of iron-regulated metabolites in the elicitation of WCS374r-mediated ISR.

In order to identify such iron-regulated bacterial traits operative in triggering ISR, we compared the potential of WCS374r to induce resistance with that of a collection of mutants deficient in the production of the siderophores pseudobactin, pseudomonine, and/or SA. All bacterial strains were routinely grown on iron-poor KB. As shown in Figure 3B, the pseudomonine-deficient mutant 4A1 induced ISR to an extent similar to that obtained after treatment with the wild-type strain, indicating that pseudomonine is not essential for WCS374r to induce resistance (Fig. 3B). Conversely, treatment with the pseudobactin-negative mutant 374-02, the pseudobactin and pseudomonine double negative mutant AT12, or the triple negative mutant BT1 no longer caused disease suppression, suggesting a pivotal role for pseudobactin in WCS374r-mediated ISR to *M. oryzae*. However, pseudobactin alone appeared to be insufficient for the onset of ISR, since we

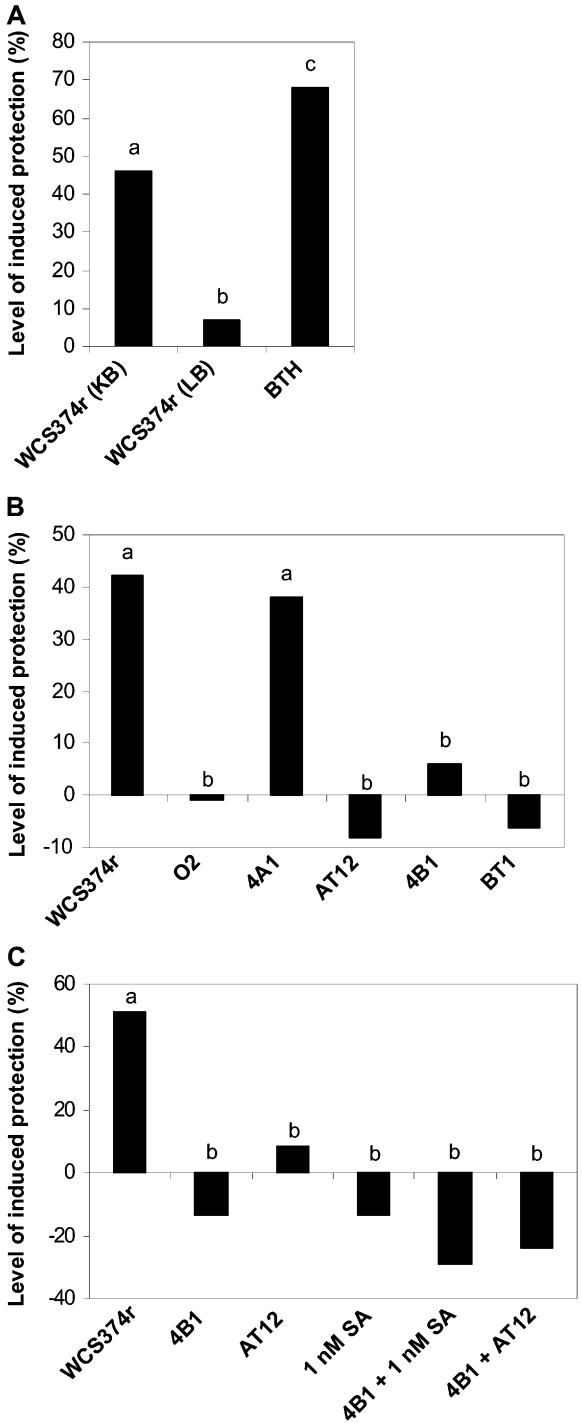

Figure 3. Influence of root treatment with *P. fluorescens* WCS374r and various mutants on rice blast (*M. oryzae*) severity. Unless otherwise stated, WCS374r and derived mutants were grown on KB and applied to rice roots and soil. Plants were challenge inoculated when 4 weeks old (five-leaf stage). For details on *M. oryzae* bioassays, see legend to Figure 1. A, Influence of the iron nutritional state of the bacterial inoculum on the level of induced resistance imparted by WCS374r. Bacteria were grown on iron-poor KB or iron-rich LB medium prior to inoculation. For comparison with chemically induced blast resistance, plants were soil drenched with BTH (0.05 mM) 3 d before challenge. B, Quantification of ISR against *M. oryzae* triggered by WCS374r and various mutant strains. Mutants derived from WCS374r have the following characteristics: 02 (Psb^-, Psm^+, SA^+), 4A1 (Psb^+, Psm^-, SA^+), AT12 (Psb^-, Psm^-, SA^+), 4B1 (Psb^+, Psm^-, SA^-), and BT1 (Psb^-, Psm^-, SA^-), where Psb = pseudobactin and Psm = pseudomonine. C, Effect of complementing the SA-deficient mutant strain 4B1 for SA production on the level of induced protection against *M. oryzae*. SA (1 nM) was applied as a soil drench 3 d before challenge. Different letters indicate statistically significant differences between treatments by Kruskal-Wallis and Mann-Whitney nonparametric tests ($\alpha = 0.05$, $n > 24$). Data presented are from representative experiments that were repeated at least twice with comparable results.

failed to observe any statistically significant differences in disease severity between treatment with the pseudobactin-positive but pseudomonine- and SA-deficient mutant 4B1 and control plants. A deficiency in root colonization could be ruled out, because bacterial counts in the rhizosphere of plants inoculated with the respective mutants were similar to those of WCS374r-treated plants (data not shown). Based on these results, we initially assumed pseudobactin to act in concert with SA in the elicitation of ISR. To test this hypothesis, we next examined the effect of inoculating roots with a mix of the ISR-deficient strains 4B1 (SA^-, Psb^+) and AT12 (SA^+, Psb^-), where Psb = pseudobactin. Alternatively, plants colonized by 4B1 were complemented with a 1 nM SA solution, a concentration equivalent to the in vitro SA production of 10^5 cfu of WCS374r. However, none of these combination treatments was able to restore ISR, making the involvement of SA and pseudobactin in ISR by WCS374r rather questionable (Fig. 3C).

SA Attenuates Pseudobactin-Induced Resistance against *M. oryzae*

To shed more light on the role of SA and pseudobactin in WCS374r-mediated ISR, we isolated pseudobactin from stationary phase cultures of WCS374r as described before (Meziane et al., 2005) and applied the purified compound, alone or in combination with SA, to the roots of hydroponically grown rice seedlings. As a positive control, plants were treated with BTH. As shown in Figure 4, purified pseudobactin applied at a concentration of 70 μg per root system increased resistance against *M. oryzae* by as much as 88%, this being similar to the level of protection induced by 0.05 mM BTH. Application of 12 μg of pseudobactin per root system was slightly less effective, as evidenced by a 67% decrease in the number of susceptible-type blast lesions. Intriguingly, hydroponic feeding of a physiologically relevant 1 nM SA solution had no marked effect on disease development, whereas coapplication of 1 nM SA and 70 μg of pseudobactin alleviated the pseudobactin-conferred protection. While indicating that pseudobactin alone suffices for full induction of WCS374r-mediated ISR to *M. oryzae*, these findings suggest negative cross talk in the direction of SA damping pseudobactin action.

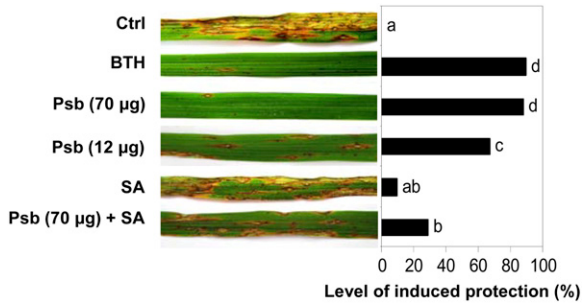

Figure 4. Effectiveness of BTH, pseudobactin (Psb), or SA-induced resistance against *M. oryzae*. To trigger resistance, rice seedlings were hydroponically fed with the various compounds by including the desired concentration in half-strength Hoagland nutrient solution at 72 h before challenge. BTH and SA were applied at concentrations of 0.05 mM and 1 nM, respectively. Pseudobactin was isolated from *P. fluorescens* WCS374r cultures and applied at a concentration of 12 or 70 μg per root system. For details on *M. oryzae* bioassays, see legend to Figure 1. Different letters indicate statistically significant differences between treatments (Kruskal-Wallis and Mann-Whitney; $\alpha = 0.05$, $n > 20$). Data presented are from a representative experiment that was repeated twice with comparable results. Ctrl, Control. [See online article for color version of this figure.]

Histochemical Analysis of Pseudobactin-Induced Resistance against *M. oryzae*

Pseudobactin Primes Rice for a Diverse Set of HR-Independent Cellular Responses

To further decipher the role of pseudobactin in WCS374r-conferred resistance in rice, we investigated the cytological and biochemical alterations associated with fungal restriction in pseudobactin-induced plants using the intact leaf sheath method developed by Koga et al. (2004). Contrary to leaf blades, leaf sheath tissue is relatively flat and optically clear, which facilitates live cell imaging, while the use of intact leaf sheaths allows the expression of numerous partial resistance responses, consistent with the continuous array of symptoms typically observed on inoculated leaf blades. Leaf sheaths of control plants and plants in which the roots were treated with either the purified pseudobactin of WCS374r (Psb374) or BTH (0.05 mM) were inoculated with a *M. oryzae* conidial suspension and sampled at 18, 24, 36, and 48 h postinoculation (hpi). Notably, microscopic assessment revealed no significant differences in the number of successful penetrations among treatments, indicating that both BTH- and Psb374-induced resistance are unlikely to impede prepenetration development by *M. oryzae* (data not shown). Starting at 36 hpi, epidermal cells were found to respond to fungal ingress through the development of various cellular reactions, which we grouped into six categories designated A to F (Fig. 5A). Type A represented infection sites showing successful fungal invasion in the absence of any obvious host response. Type B reactions, on the other hand, were characterized by a pale yellow or brown discoloration of the anticlinal cell walls and weakly enhanced vesicular activity. Epidermal sites in which the invasive hyphae were confined to the primary penetrated cell due to expression of the so-called whole plant-specific resistance (WPSR; Koga et al., 2004), a type of age-related resistance characterized by the occurrence of large, brownish granules in the cytoplasm, were scored as type C. Infection type D likewise comprised single-cell infection sites but was associated with intense browning of the anticlinal cell walls and the occurrence of round and tubular vesicles in the cytoplasm. Epidermal cells classified as type E displayed a remarkable interaction phenotype in which fungal growth was curtailed shortly after penetration by means of infection hyphae-encasing tubers, the nature of which is still elusive, as staining with phloroglucinol provided no compelling evidence for the involvement of lignin-derived deposits. Finally, type F represented a HR-like reaction, as evidenced by dense granulation of the cytoplasm and a bright autofluorescence of the epidermal cell walls. An overview of the temporal changes in the frequency of the various cellular reaction types is presented in Figure 5B. At 36 hpi, control plants almost exclusively displayed type A reactions (up to 92% of all interactions). A decrease in type A reaction from 36 to 48 hpi was accompanied by a drastic increase in the frequency of appressorial sites exhibiting a type B reaction, reaching a level of 55% by 48 hpi. BTH-induced resistance, on the other hand, was characterized by a high frequency of interaction sites with attacked cells expressing HR-related type F reaction (70% of all interactions), resulting in abrupt arrest of fungal proliferation. Most conspicuously, Psb374-supplied plants showed a strikingly different profile of effector responses in that they did not develop any HR-like responses but rather mounted type D and type E reactions, accounting for 33% and 50% of all interactions by 48 hpi, respectively. Together, these observations suggest that Psb374 primes rice for a diverse set of HR-independent cellular defenses.

Pseudobactin Primes Rice for Enhanced Pathogenesis-Related Hydrogen Peroxide Formation

Production of reactive oxygen species during the oxidative burst is one of the most peculiar defense responses in plant-pathogen interactions; therefore, we next compared pathogenesis-related hydrogen peroxide (H_2O_2) generation using 3,3′-diaminobenzidine (DAB) staining. In this endogenous peroxidase-dependent assay, reddish-brown precipitates are deposited at the sites of H_2O_2 accumulation (Thordal-Christensen et al., 1997). Consistent differences between treatments were seen from 24 hpi onward. At this time, approximately one-fourth of all Psb374- or BTH-treated epidermal cells adjacent to fungal appressoria showed a local brownish staining of the anticlinal walls, whereas little staining was evident in the sheaths of control plants (data not shown). Impor-

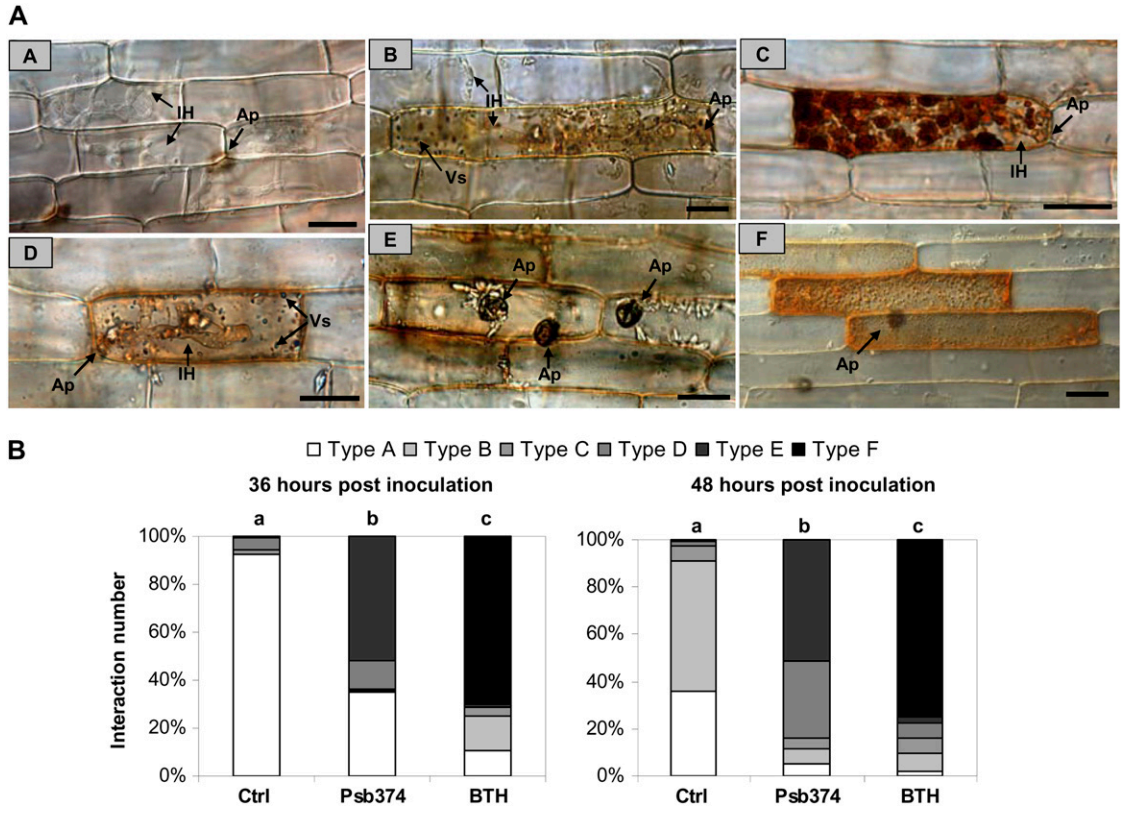

Figure 5. Quantitative cytological analysis of cellular responses in leaf sheath epidermal cells of control (Ctrl), BTH-pretreated, and pseudobactin-pretreated rice plants infected with *M. oryzae*. Roots of young hydroponically grown rice seedlings (6.5-leaf stage) were treated with either the purified pseudobactin of *P. fluorescens* WCS374r (Psb374; 70 μg per plant) or BTH (0.05 mM); 3 d later, plants were challenged by injecting the intact leaf sheaths with a conidial suspension of *M. oryzae*. A, Single-cell interaction phenotypes were grouped into six categories, designated A to F. Micrographs depict representative examples: category A, vigorous invasion of living tissues in the absence of visible host responses; category B, occurrence of cytoplasmic vesicles and slight browning of the anticlinal walls of the first-invaded epidermal cell following fungal invasion of neighboring cells; category C, epidermal cells expressing so-called WPSR (Koga et al., 2004) as indicated by the presence of large orange-brown granules in the cytoplasm; category D, restriction of fungal development to the first-invaded epidermal cell associated with intense browning of anticlinal epidermal cell walls and enhanced vesicular activity; category E, development of invading hyphae-embedding tubules confers prompt fungal arrest in Psb374-induced epidermal cells; category F, BTH-specified HR-like reaction characterized by dense cytoplasmic granulation. Ap, Appressorium or appressorial site; IH, invading hyphae; Vs, vesicles. Bars = 20 μm. B, Frequency distribution of the above-mentioned interaction phenotypes at 36 and 48 hpi. Each bar represents the mean of eight replications stemming from four plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment are presented. Repetition of experiments led to results very similar to those shown. Bars with the same letter are not significantly different according to Kruskal-Wallis and Mann-Whitney comparison tests at $\alpha = 0.05$.

tantly, ascorbate treatment of inoculated leaf sheaths abolished staining at the respective sites, confirming the specificity of the staining for H_2O_2 accumulation. Local DAB staining of anticlinal cell walls disappeared within 36 hpi, when the fungus had started to develop branched, bulbous invading hyphae. From this time onward, different patterns of DAB staining could be distinguished (depicted at 48 hpi in Fig. 6A). Interestingly, both the susceptibility-related infection type A, in which fungal hyphae vigorously invaded living tissue, and the Psb374-specified infection type D, characterized by lignituber-like structures encasing invasive hyphae, remained essentially free of DAB accumulation; these reactions we designated DAB type I and type II, respectively. Conversely, in some cases, H_2O_2 accumulated in the primary invaded cell following spread of the invading hyphae into neighboring cells (type III). Restriction of hyphal growth to the initially invaded cell was associated with variable patterns of DAB staining. In some cases, cells were filled with numerous DAB-positive vesicle-like bodies targeted to the invading hyphae (type IV), whereas in WPSR- and HR-expressing cells, H_2O_2 typically accumulated within the characteristic cytoplasmic aggregation (types V and VI). Finally, in a limited number of cases, abrupt arrest of fungal ingress coincided with

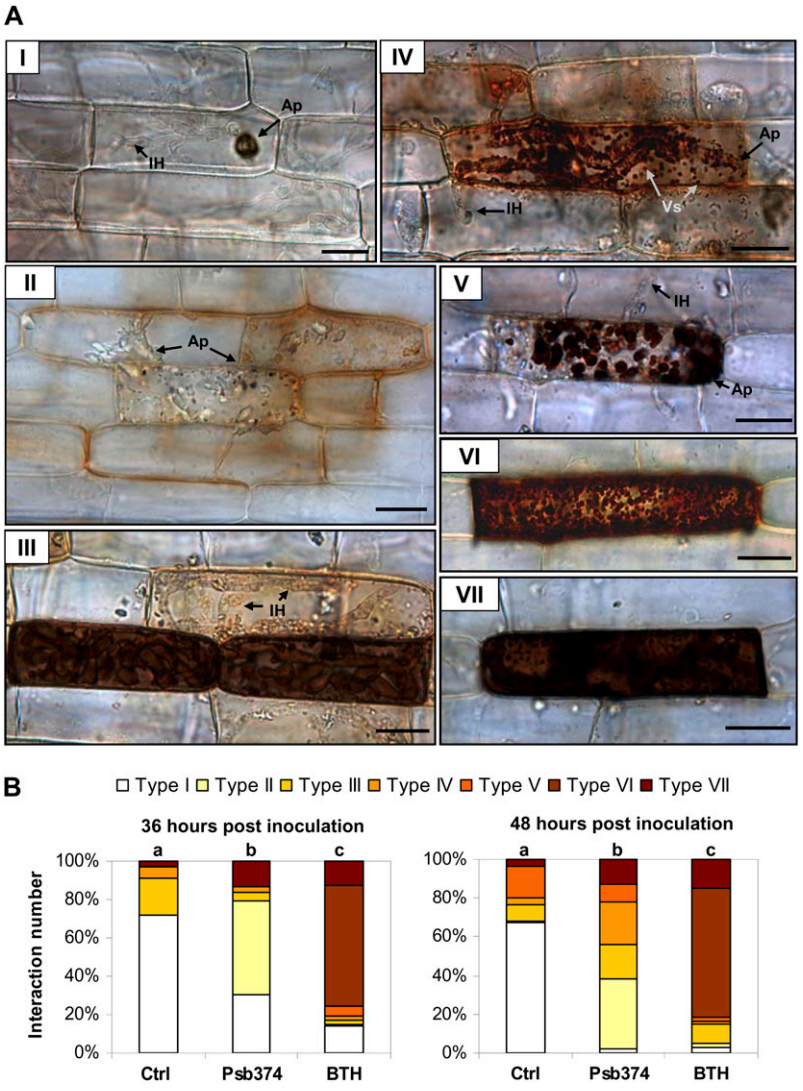

Figure 6. Effects of pseudobactin and BTH pretreatment on H_2O_2 accumulation in epidermal rice sheath cells inoculated with *M. oryzae*. Roots of young hydroponically grown seedlings (6.5-leaf stage) were treated with either the purified pseudobactin of *P. fluorescens* WCS374r (Psb374; 70 μg per plant) or BTH (0.05 mM); 3 d later, plants were challenged by injecting the intact leaf sheaths with a conidial suspension of *M. oryzae*. A, Micrographs depicting examples of distinct H_2O_2 accumulation patterns in inoculated leaf sheaths supplied with DAB: I, successful fungal colonization of living control (Ctrl) cells, with no DAB staining visible; II, Psb374-specified fungal arrest in the primary invaded cell (Fig. 5A, category E) is not associated with any detectable H_2O_2 accumulation; note the difference between the natural browning of attacked cells (this image) and the intense reddish-brown coloration due to DAB staining of H_2O_2 (images III–VII); III, DAB accumulation in a primary epidermal cell following fungal invasion of adjacent cells; IV, accumulation of DAB-positive vesicle-like bodies in the vicinity of the invasive hyphae; V, WPSR-expressing cells (for details, see legend to Fig. 5) filled with DAB-stained granules; VI, DAB-positive cytoplasmic granules in BTH-treated hypersensitively reacting cells; VII, whole-cell DAB staining. Ap, Appressorium or appressorial site; IH, invading hyphae; Vs, vesicles. Bars = 20 μm. B, Frequency distribution of the above-mentioned DAB staining patterns at 36 and 48 hpi. Each bar represents the mean of eight replications stemming from four plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment are presented. Repetition of experiments led to results very similar to those shown. Bars with the same letter are not significantly different according to Kruskal-Wallis and Mann-Whitney comparison tests at $\alpha = 0.05$.

massive H_2O_2 accumulation in the entire cell, beginning as early as 30 hpi (type VII). A comparative kinetic analysis of H_2O_2 formation revealed that by 36 hpi, approximately 75% of all interaction sites in both control and Psb374-treated tissue lacked any DAB-detectable H_2O_2 (Fig. 6B). However, whereas in control cells the absence of H_2O_2 accumulation at this time point was related to successful fungal colonization, lack of DAB staining in Psb374-induced tissue mainly resulted from the high ratio of appressorial sites exhibiting pathogen-blocking type E reactions. Hence, the high frequency of DAB-negative interaction sites in control and Psb374-treated plants reflects distinct cellular responses with dramatically different outcomes. By 48 hpi, the overall proportion of sites that showed DAB staining was significantly higher in Psb374-treated plants than in control plants. In Psb374-induced tissue, a strong decline in the frequency of DAB-negative type I cells from 36 to 48 hpi corresponded to an approximately 10% increase in types III, IV, and V reactions, whereas in control plants, the number of types I and III reactions decreased very slowly at a rate corresponding to an increase in the number of type V reactions. Compared with the well-restricted H_2O_2 production in Psb374-supplied sheath cells, BTH-triggered HR was associated with a massive oxidative burst (type VI) beginning as early as 30 hpi, suggesting that the mechanism(s) by which BTH boosts pathogen-triggered H_2O_2 generation may be different from Psb374-conditioned priming. Starting at 52 hpi, a strong accumulation of H_2O_2 was found in control mesophyll cells that appeared to collapse, whereas in Psb374- and BTH-treated plants, DAB staining in the mesophyll tissue was only rarely observed (data not shown). However, at these late infection stages, massive H_2O_2 accumulation most likely reflects deregulated cell physiology and overtaxed antioxidative capacities, rather than a controlled defense response that restricts cellular accessibility for *M. oryzae*. Taken together, these results indicate that Psb374-mediated resistance

against *M. oryzae* involves a timely, highly localized, and well-restricted production of H_2O_2 in the epidermis.

Pseudobactin-Induced Resistance against M. oryzae Is Associated with Priming for Accelerated Cell Wall Fortification

Because H_2O_2 is often used as a substrate for peroxidase-dependent cross-linking of cell wall polymers, different staining procedures were performed to visualize changes in the cell wall. Cross-linking of cell wall proteins was detected with Coomassie Brilliant Blue subsequent to protein denaturation and free protein removal (Mellersh et al., 2002), whereas safranin-O was used to detect the peroxidative incorporation of phenolic compounds in the cell wall, a fortification mechanism important during lignification and suberization (Lucena et al., 2003). As shown in Figure 7, cell wall modification was more abundant and appeared earlier in Psb374- and BTH-treated plants than in the control plants; starting from 24 hpi, the anticlinal walls of Psb374- or BTH-induced epidermal cells showed intense safranin staining, whereas in control plants, staining was weak and only detectable in limited zones of the anticlinal walls of a few colonized cells from 36 hpi onward (Fig. 7A). Likewise, protein cross-linking was seldom detected prior to 48 hpi in control plants, whereas in Psb374-supplied or BTH-induced cells, it was evident in the anticlinal and/or periclinal walls of most interaction sites at 36 hpi (Fig. 7B). Similar results were obtained when assaying for autofluorescence, the early occurrence of which is considered a hallmark of rice defense against *M. oryzae* (Rodrigues et al., 2005). Although autofluorescence was detectable as early as 18 hpi regardless of the treatment, from this time onward, the frequency of autofluorescent appressorial sites increased much

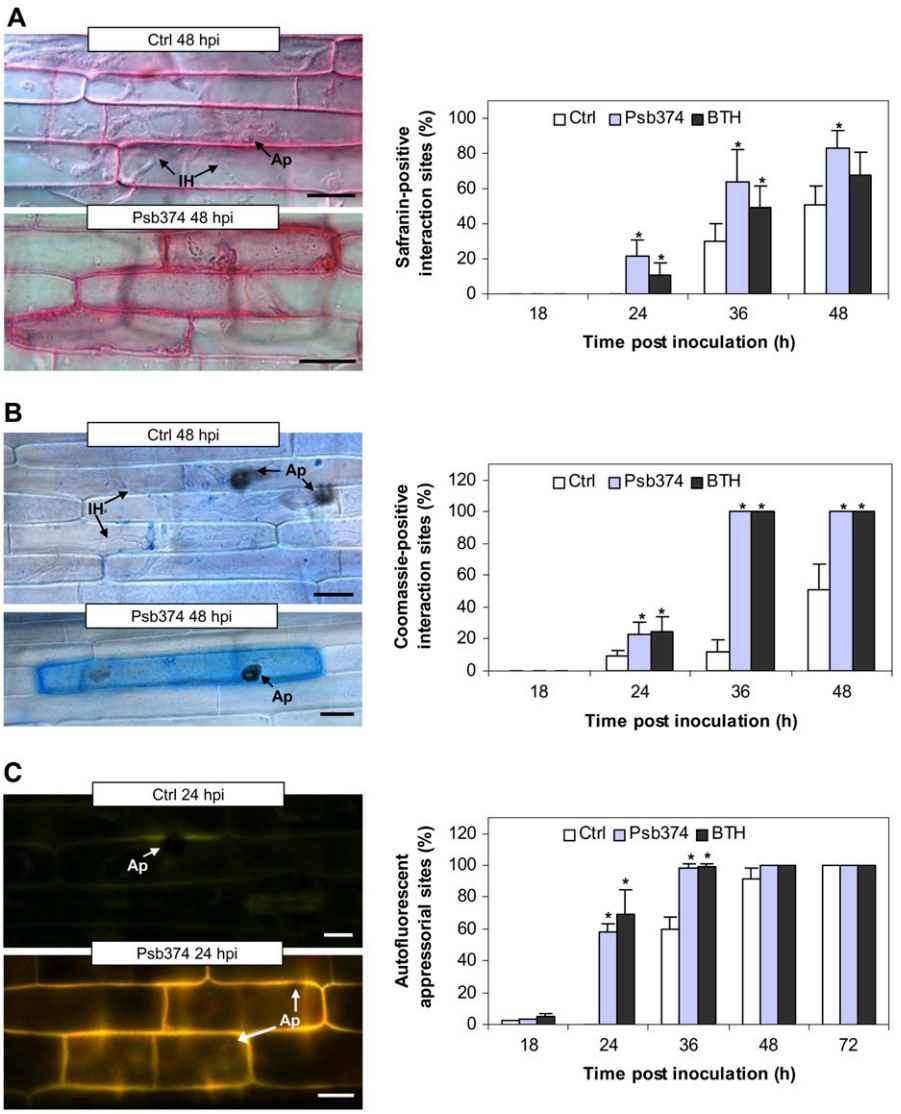

Figure 7. Quantitative cytological analysis of cell wall modifications in control (Ctrl), BTH-pretreated, and pseudobactin-pretreated rice plants challenged with *M. oryzae*. Roots of young hydroponically grown rice seedlings (6.5-leaf stage) were treated with either the purified pseudobactin of *P. fluorescens* WCS374r (Psb374; 70 μg per plant) or BTH (0.05 mM); 3 d later, plants were challenged by injecting the intact leaf sheaths with a conidial suspension of *M. oryzae*. A and B, Priming of pathogen-induced cell wall reinforcements in Psb374-treated plants. Peroxidative incorporation of phenolics compound and protein cross-linking were visualized with safranin-O (red-pink; A) and Coomassie Brilliant Blue (dark blue; B), respectively. C, Left, Representative epifluorescence images of control and Psb374-supplied epidermal cells at 24 hpi (blue light excitation). Right, Psb374 and BTH prime rice for accelerated deposition of autofluorogenic phenolics at sites of attempted pathogen entry. Asterisks indicate statistically significant differences compared with the noninduced control treatment. Each bar represents the mean and SD of six replications stemming from three plants. At least 50 single-cell interaction sites originating from representative sheath sections were examined per replication. Data from one experiment are presented. Repetition of experiments led to results very similar to those shown. Bars = 20 μm.

more rapidly in Psb374- or BTH-treated plants than in nontreated control plants, indicating that both inducers prime rice for augmented deposition of phenolic compounds at sites of attempted pathogen entry (Fig. 7C). Conceivably, enrichment of the host cell wall with phenolics contributes to the elaboration of permeability barriers that prevent pathogen spread and enzymatic cell wall degradation.

Exogenous SA Abrogates Psb374-Induced Priming

The observation that Psb374-pretreated plants exhibited potentiated expression of multiple cellular defense responses suggested that priming for enhanced basal defense might constitute a crucial facet of the Psb374-induced resistance response. To test this hypothesis, we next examined the effect of exogenous SA application on the manifestation of Psb374-induced priming. As illustrated in Table I, coapplication of 1 nM SA with 70 μg of Psb374 significantly decreased the frequency of Psb374-specified type E reactions (i.e. infection-blocking tubules). Adding SA to the Psb374 solution also perturbed the early occurrence of DAB staining and autofluorescence in Psb374-treated tissues and alleviated Psb374-primed protein cross-linking and cell wall fortification. Along with the suppressive effect of coapplied SA on the level of Psb374-induced protection against *M. oryzae* (Fig. 4), these results indicate that Psb374-triggered ISR is based on priming for enhanced expression of an attacker-induced multifaceted cellular defense program.

Psb374 Antagonizes Pathogen-Induced Activation of SA-Responsive *PR* Genes

The results above, together with the *PR* gene independence of ISR in Arabidopsis (Pieterse et al., 1996), prompted us to investigate whether Psb374 pretreatment also affects *PR* transcript accumulation. To this end, we tested control, BTH-induced, and Psb374-supplied plants for expression of the rice *PR*-like genes *OsPR1b* and *PBZ1/PR10a*. Both of these genes are known to be responsive to *M. oryzae* infection (Kim et al., 2001) and have recently been implicated in the BTH-inducible and SA-mediated signaling branch of the rice defense network (Shimono et al., 2007). Quantitative reverse transcription (RT)-PCR analysis revealed that neither BTH application nor Psb374 treatment alone significantly altered *OsPR1b* or *PBZ1* mRNA accumulation at any of the time points investigated (Fig. 8A; data not shown). However, significant differences between treatments became evident when challenging with *M. oryzae*. In accordance with previous reports (Midoh and Iwata, 1996; Yang et al., 2004), *PBZ1* transcript levels responded strongly to blast infection, showing an approximately 250-fold induction relative to mock-inoculated controls by 48 hpi (Fig. 8A). Interestingly, application of Psb374 prior to inoculation attenuated this pathogen-induced activation of *PBZ1*, whereas pretreatment with BTH caused a faster and stronger induction of the latter gene in comparison with the expression measured in challenged, noninduced plants (Fig. 8A). Transcript accumulation of the *OsPR1b* gene mirrored the profile observed for *PBZ1* (Fig. 8B), suggesting that Psb374 antagonizes *M. oryzae*-induced transcription of SA-responsive *PR* genes.

DISCUSSION

ISR is a phenomenon whereby disease resistance against subsequent microbial infection is induced at the whole plant level in response to colonization of the roots by certain plant growth-promoting rhizobacteria. Compared with the relative wealth of information in experimentally tractable plant species such as Arabidopsis, our understanding of the molecular mechanisms underlying ISR in economically important cereal crops is still in its infancy. In this work, we have focused on the bacterial determinants and host defense responses underlying rhizobacteria-activated ISR in rice, the most important food source worldwide

Table I. *Influence of SA coapplication on Psb374-induced defense priming*

The data represent means ± SD of four replicates of each 100 interaction sites per leaf sheath. Each of three independent experiments gave very similar results. Within each row, different letters indicate statistically significant differences between treatments (Fisher's LSD test; $\alpha = 0.05$).

Reaction	Treatment[a]			
	Control	SA	Psb374	SA + Psb374
		% of interaction sites		
Infection-blocking tubules (36 hpi)[b]	Not seen	Not seen	43.3 ± 18.6b	7.5 ± 2.4a
DAB staining (24 hpi)[c]	Not seen	Not seen	26.2 ± 8.7b	5.6 ± 2.1a
Autofluorescence (24 hpi)	2.1 ± 0.8a	9.4 ± 4.2b	66.8 ± 10.6c	18.6 ± 6.7b
Coomassie Brilliant Blue staining (36 hpi)	24.6 ± 5.8a,b	19.2 ± 4.6a	94.2 ± 5.2c	32.4 ± 9.2b
Safranin staining (36 hpi)	29.5 ± 10.2a	24.6 ± 6.8a	72.6 ± 8.6b	21.4 ± 11.3a

[a]SA (1 nM) and purified WCS374r pseudobactin (Psb374; 70 μg per root system) were applied either alone or in combination to the roots of hydroponically grown rice seedlings (6.5-leaf stage) at 3 d prior to challenge with *M. oryzae*. [b]Interaction phenotype E as described in legend to Figure 5. [c]Percentage of interaction sites associated with reddish-brown precipitates in the anticlinal cell wall.

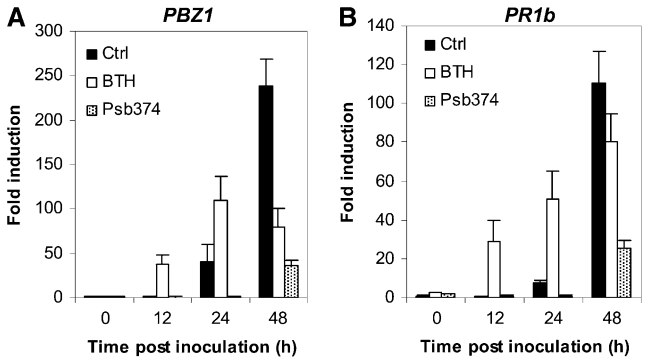

Figure 8. Effects of BTH and Psb374 pretreatment on *PBZ1/PR10* (A) and *OsPR1b* (B) transcript accumulation in *M. oryzae*-infected rice leaves. BTH (0.05 mM) or Psb374 (70 μg per root system) was applied to the roots of 4-week-old rice plants 3 d before challenge. At the indicated time points after inoculation, fully expanded fourth leaves from six plants were harvested, pooled, and subjected to quantitative RT-PCR analysis. Gene expression levels were normalized using actin (Os03g50890) as an internal reference and calculated relative to the expression in mock-treated control (Ctrl) plants at 0 h. Data presented are means ± SD of three replicates from a representative experiment. Two series of independent experiments were carried out giving reproducible results.

and a pivotal model for molecular genetic studies of disease resistance in monocotyledonous plants. We show that colonization of the roots of rice by the well-characterized biocontrol agent *P. fluorescens* WCS374r renders foliar tissues more resistant to rice blast disease, caused by the heterothallic ascomycete *M. oryzae* (Fig. 1). Our data also reveal that this WCS374r-ISR is not based on direct activation of basal resistance mechanisms but rather acts through pseudobactin-mediated priming for a pronounced multifaceted cellular defense program (Figs. 3–7). Moreover, we demonstrate that ISR by WCS374r requires components of the ET and JA pathways, rather then SA accumulation or enhanced *PR* gene expression, suggesting that rice might have evolved a disease resistance pathway similar to the classic ISR pathway in Arabidopsis (Figs. 2 and 8).

Pseudobactin: Iron-Chelating Protagonist in the Initiation of *P. fluorescens* WCS374r-Mediated ISR

To date, several bacterial traits have been implicated in the initiation of WCS374r-ISR, including the *O*-antigenic side chain of outer membrane lipopolysaccharides, SA, and the siderophore pseudobactin (Leeman et al., 1995, 1996; Ran et al., 2005a, 2005b). In this study, we initially assumed pseudobactin and SA to be corequired for the initiation of ISR against *M. oryzae*, based on the observation that both the pseudobactin-deficient mutant 374-02 and the pseudobactin-proficient, yet SA-negative, mutant 4B1 lost the ability to mount ISR (Fig. 3). However, contradictory results were obtained when testing the isolated bacterial compounds: purified WCS374r-derived pseudobactin (Psb374) triggered high levels of resistance against rice blast, whereas exogenous SA failed to cause any substantial disease reduction (Fig. 4), indicating that Psb374 alone suffices for induction of ISR. Moreover, coapplication of SA with Psb374 attenuated the Psb374-induced resistance, prompting the question of how WCS374r bacteria, which simultaneously produce both of these metabolites in vitro, are able to trigger ISR. One likely scenario originates from the observation that treatment with WCS374r does not induce systemic resistance in Arabidopsis against *P. syringae* pv *tomato*, whereas the application of SA does (Van Wees et al., 1997). This discrepancy suggests that SA produced by WCS374r is not exuded into the rhizosphere, which may be due to the fact that upon iron limitation WCS374r produces not only pseudobactin and SA but also pseudomonine, a siderophore containing a SA moiety (Mercado-Blanco et al., 2001). Accordingly, it is plausible that in the rhizosphere, where iron-limiting conditions tend to prevail, all WCS374r-produced SA is channeled into pseudomonine that does not antagonize pseudobactin action. Nonetheless, if pseudobactin is the crucial determinant of WCS374r-ISR against *M. oryzae*, a question remains concerning the ISR-minus phenotype of the pseudobactin-positive mutant 4B1. One possible explanation for these conflicting observations lies in the fact that mutant 4B1 was constructed by gene replacement of the native *psmB* gene. *pmsB* encodes a presumed isochorismate-pyruvate lyase that catalyzes the conversion of isochorismate to pyruvate and SA (Djavaheri, 2007). Interestingly, recent evidence suggests that, in line with its closest homolog in *P. aeruginosa*, the catalytically promiscuous SA biosynthesis protein PchB, PmsB not only possesses isochorismate-pyruvate lyase but also chorismate mutase activity (Kunzler et al., 2005). Since chorismate mutase is located at the branch point of the shikimate pathway leading to the biosynthesis of Tyr and Phe, the enzyme constitutes a key point of regulation for maintaining the correct balance of aromatic amino acids in the cell (Neuenschwander et al., 2007). Hence, it can be envisaged that a mutation in such a regulatory enzyme might have a pleiotropic effect hampering the induction of ISR. Alternatively, mutant 4B1 might simply produce too little pseudobactin in the rhizosphere to be effective at inducing resistance.

Rice and Arabidopsis Share Conserved Disease Resistance Pathways

In many dicot plants, the role of SA as a global multicomponent regulator of various inducible defense responses is well established (Loake and Grant, 2007). Following pathogen infection, endogenous levels of SA and its conjugates increase dramatically, preceding the induction of *PR* genes and the onset of local resistance and SAR (Durrant and Dong, 2004). In

rice, however, the role of SA is still a matter of debate. Rice differs from most other plants in that it contains very high basal levels of endogenous SA that are not elevated further in response to pathogen infection, making the role of the SA signaling pathway in rice disputable (Silverman et al., 1995). A number of recent reports, however, do support an active role for a BTH-inducible and WRKY45- or NPR1-regulated SA signaling pathway in the rice defense response (Chern et al., 2001, 2005; Fitzgerald et al., 2004; Shimono et al., 2007; Yuan et al., 2007). Emerging from these studies is the view that rice, in spite of its high constitutive SA levels, has evolved a SA-mediated SAR pathway similar to that in Arabidopsis. In this study, we provide, to our knowledge, the first report of a similar phenomenon with regard to rhizobacteria-induced resistance signaling. ISR bioassays with SA-nonaccumulating NahG plants (Yang et al., 2004), the ET-insensitive *OsEIN2* antisense line 471 (Jun et al., 2004), and the JA biosynthesis mutant *hebiba* (Riemann et al., 2003) revealed that WCS374r-mediated ISR against *M. oryzae* functions independently of SA but requires intact responsiveness to ET as well as a functional JA pathway (Fig. 2). In this respect, WCS374r-ISR against *M. oryzae* mirrors classic WCS417r-elicited ISR in Arabidopsis (Pieterse et al., 1996, 1998). Consistent with this is the finding that treatment with Psb374, which faithfully mimics WCS374r in activating ISR, does not lead to direct transcriptional activation or priming of SA-inducible *PR* genes, such as *OsPR1b* and *PBZ1* (Fig. 8). In contrast to WCS374r-ISR, but similar to BTH-induced resistance in dicots (Friedrich et al., 1996; Gorlach et al., 1996), chemical induction of blast resistance by exogenous application of BTH was fully retained in 471, *hebiba*, and NahG rice plants and involved the potentiation of SA-inducible gene expression (Figs. 2 and 8). Taken together, these results not only reinforce the contention that rice is endowed with a BTH-inducible SAR-like resistance pathway (Shimono et al., 2007; Yuan et al., 2007) but also hint at a conserved mechanism for ISR signaling in rice and Arabidopsis. It is noteworthy, however, that unlike WCS374r-ISR, induction of systemic resistance against *M. oryzae* by *P. aeruginosa* 7NSK2 was found to be SA-dependent (D. De Vleesschauwer and M. Höfte, unpublished data), indicating that the signal transduction pathway governing rhizobacteria-mediated ISR against *M. oryzae* at least in part depends on the eliciting bacterium. Nonetheless, the apparent similarities between WCS374r- and WCS417r-activated ISR signaling in rice and Arabidopsis, respectively, support and further extend the earlier notion of ancient plant-inducible defense pathways that are shared between monocots and dicots (Morris et al., 1998). This notion, however, does not rule out the possibility that individual plant species may differ in the fine-tuned regulation of such conserved defense pathways. For instance, while ectopic expression of a rice NPR1 homolog induces constitutive activation of SA-responsive *PR* gene expression and provokes spontaneous development of a lesion mimic/cell death phenotype (Chern et al., 2005), none of these reactions is evident in NPR1-overexpressing Arabidopsis until treatment with SAR inducers or pathogen infection (Cao et al., 1998). Such species-specific regulation of conserved plant defense mechanisms may also apply to ISR-associated resistance phenomena. Indeed, whereas the impaired ISR response of JA-deficient *hebiba* argues that in rice WCS374r-ISR develops coincidently with increases in endogenous JA content (Fig. 2), in Arabidopsis neither induction nor expression of WCS417r-ISR was found to be associated with substantial alterations in JA biosynthesis (Pieterse et al., 2000). Instead, recent evidence indicates that elicitation of WCS417r-ISR sensitizes Arabidopsis for the perception of attacker-induced JA (Pozo et al., 2008). Hence, although rice and Arabidopsis appear to share a conserved ISR pathway, the modulation of this JA-dependent resistance conduit may be quite divergent. To our interest, the significance of elevated JA levels in mediating rice disease resistance was recently highlighted by the enhanced blast resistance of transgenic rice plants overexpressing allene oxide synthase, a key enzyme in the JA biosynthetic pathway (Mei et al., 2006).

Parallels between WCS374r-Mediated ISR and Wound-Inducible Systemic Resistance against *M. oryzae*

The predicted role of JA in WCS374r-ISR is reminiscent of the situation in wounded rice plants, where systemic resistance against *M. oryzae* is preceded by a strong and transient accumulation of nonconjugated JA in local and systemic tissues (Schweizer et al., 1998). Wound-inducible blast resistance further resembles WCS374r-ISR in that it delivers a similar level of systemic protection without the customary *PR* gene induction and is likewise abrogated in mutant *hebiba* plants (Schweizer et al., 1998; Riemann et al., 2003; D. De Vleesschauwer and M. Höfte, unpublished data). Regarding these similarities between WCS374r-ISR and wound-induced resistance, it is tempting to speculate that both phenomena are based on similar resistance mechanisms. Such a concept would also provide a mechanistic framework for the attenuation of SA-responsive *PR* gene expression in challenged Psb374-induced plants (Fig. 8). In some interesting work on rice responding to mechanical wounding, Lee et al. (2004) demonstrated that JA-induced depletion of endogenous SA levels constitutes an important regulatory mechanism for JA antagonism of SA signaling. In this scenario, if the establishment of WCS374r- and Psb374-mediated ISR coincides with a JA burst, the inverse correlation between endogenous JA and SA may account for the down-regulation of at least *PR1b*, the induction of which is considered to be a reliable marker for activation of the SA-regulated defense pathway in rice (Yuan et al., 2007). In a similar vein, antagonistic cross talk between SA and JA signaling may also explain the inhibitory effect of exogenous SA on the Psb374-provoked resistance against *M. oryzae*

(Fig. 4; Table I). Antagonistic cross-communication between the SA and JA pathways in rice was recently shown to be orchestrated by OsWRKY13, a WRKY transcription factor functioning upstream of the rice NPR1 homolog OsNH1 (Qiu et al., 2007; Yuan et al., 2007). Ectopic expression of *OsWRKY13* represses JA biosynthetic genes while activating a specific subset of SA-dependent genes, suggesting that OsWRKY13 antagonizes JA-dependent defenses by negative feedback regulation of JA biosynthesis (Qiu et al., 2007). Whether *OsWRKY13* expression is altered in ISR-expressing plants is currently being investigated.

WCS374r-Triggered ISR Is Based on Pseudobactin-Mediated Priming for a Multifaceted Cellular Defense Response

In common with many other investigations (Benhamou et al., 1996; Ahn et al., 2002, 2007; Kim et al., 2004; Verhagen et al., 2004; Tjamos et al., 2005), our results support the view that rhizobacteria-mediated ISR is not based on direct activation of defense mechanisms but rather results from a sensitization of the tissue to express basal defenses faster and/or more strongly upon subsequent pathogen attack. Such a priming effect was borne out by the observation that challenge inoculation of Psb374-induced plants with *M. oryzae* entailed the prompt expression of a pronounced multifaceted cellular defense program, comprising rapid recruitment of phenolic compounds at sites of attempted pathogen entry, elaboration of specific sheath cell reactions, and a timely oxidative burst putatively driving cell wall fortification and protein cross-linking (Figs. 5–7). The importance of defense priming in the Psb374-activated resistance mechanism was shown by the effect of adding SA to the Psb374 feeding solution, which not only counteracted the distinct Psb374-primed cellular responses but concurrently alleviated Psb374-provoked resistance against *M. oryzae* (Fig. 4; Table I). In conjunction with the strict pseudobactin dependence of WCS374r-ISR, such close correlation between the manifestation of priming and the establishment of Psb374-induced resistance suggests that Psb374-mediated priming for enhanced defense may constitute the in situ mechanism underpinning WCS374r-ISR against *M. oryzae*. Hence, it is not inconceivable that WCS374r bacteria protect rice from *M. oryzae* by releasing pseudobactin-type siderophores into the rhizosphere, thereby inducing a prealerted state of defense enabling plants to respond better and more rapidly to subsequently inoculated pathogens. In line with this concept, we previously uncovered priming as a crucial facet of the resistance mechanism underlying *P. aeruginosa* 7NSK2-mediated ISR against *M. oryzae*. Feeding rice plants with the redox-active pigment pyocyanin, the crucial determinant of 7NSK2-mediated ISR, resulted in enhanced attacker-induced HR-like cell death in naive leaves, a phenomenon shown to be orchestrated by reiterative H_2O_2 microbursts (De Vleesschauwer et al., 2006). Interestingly, similar phenocopies of hypersensitively dying epidermal cells in the vicinity of fungal hyphae were evident in challenged rice plants pretreated with BTH (Fig. 5), suggesting that BTH and pyocyanin might feed into a similar resistance pathway. Psb374-elicited ISR, on the other hand, was not associated with HR-like cell death but involved the potentiation of a coordinated set of distinct cellular reactions, the fast manifestation of pathogen-blocking tubules being a prominent component (Fig. 5). In combination with our unpublished findings (D. De Vleesschauwer and M. Höfte, unpublished data) that WCS374r-ISR and 7NSK2-ISR differ in their requirement for SA, these results support the notion that WCS374r and 7NSK2 bacteria employ distinct strategies to mount ISR and suggest that rice is endowed with multiple, at least partly distinct, blast-effective resistance pathways. This conclusion is further supported by a large body of evidence demonstrating minimal overlap in the gene sets activated by different blast resistance inducers (Midoh and Iwata, 1996; Schweizer et al., 1997, 1999; Nakashita et al., 2003; Tanabe et al., 2006). We also tested resistance induced by pyocyanin or pseudobactin against the necrotrophic pathogens *Rhizoctonia solani* and *Cochliobolus miyabeanus*. However, in both cases, no protection was obtained, suggesting that the defense responses triggered by the latter elicitors are not effective against necrotrophic pathogen assault (De Vleesschauwer et al., 2006; D. De Vleesschauwer and M. Höfte, unpublished data).

Table II. *Bacterial strains used in this study with their relevant characteristics*

Km, Kanamycin; Pch, pyochelin; Psb, pseudobactin; Psm, pseudomonine; Pvd, pyoverdine; superscript r, resistant; Rif, rifampycin; Tc, tetracycline.

Strains	Relevant Characteristics	Reference or Source
WCS374r	Psb^+, Psm^+, SA^+, spontaneous Rif^r mutant of WCS374; Rif^r	Geels and Schippers (1983)
374-02	Psb^-, Psm^+, SA^+, Tn5 transposon mutant of WCS374; Km^r	Weisbeek et al. (1986)
4A1	Psb^+, Psm^-, SA^+, *pmsA* mutant of WCS374r obtained by site-directed mutagenesis; Rif^r, Km^r	Djavaheri (2007)
AT12	Psb^-, Psm^-, SA^+, Tn5 transposon mutant of 4A1; Rif^r, Km^r, Tc^r	Djavaheri (2007)
4B1	Psb^+, Psm^-, SA^-, *pmsB* mutant of WCS374r obtained by site-directed mutagenesis; Rif^r, Km^r	Djavaheri (2007)
BT1	Psb^-, Psm^-, SA^-, Tn5 transposon mutant of 4B1; Rif^r, Km^r, Tc^r	Djavaheri (2007)

CONCLUSION

In summary, we have shown that colonization of the roots of rice by pseudobactin-producing WCS374r bacteria sensitizes naive leaves for potentiated expression of a multifaceted cellular defense response, resulting in an enhanced level of resistance against the leaf blast pathogen *M. oryzae*. Our results also provide evidence for a WCS374r-activated signaling conduit in rice similar to the classic SA-independent but JA/ET-dependent signal transduction pathway controlling rhizobacteria-mediated ISR in Arabidopsis. Furthermore, it is evident from this study that WCS374r triggers a resistance that is mechanistically different from BTH-inducible blast resistance as well as systemic resistance induced by *P. aeruginosa* 7NSK2, suggesting the coexistence of multiple pathways leading to induced resistance against *M. oryzae*. Further elucidation of the bacterial traits and dynamic host responses underpinning rhizobacteria-mediated ISR in rice will not only advance our fundamental understanding of how rice plants cope with enemies in the context of induced resistance but also may be instrumental in developing new strategies for biologically based, environmentally friendly, and durable disease control in economically important cereal crops.

MATERIALS AND METHODS

Plant Materials

The highly susceptible rice (*Oryza sativa indica*) cv CO39 was routinely used in this study. Transgenic NahG rice and its parental line, *japonica* cv Nipponbare, were a kind gift from Dr. Yinong Yang (Pennsylvania State University). Seeds of cv Dongyin and the transgenic line 471, expressing the *OsEIN2* antisense construct, were kindly provided by Dr. Gynheung An (Yonsei University), while JA-deficient *hebiba* mutant seeds and the corresponding wild type, *japonica* cv Nihonmasari, were a kind gift from Dr. Peter Nick (Karlsruhe University). Unless otherwise noted, rice plants were grown on soil under greenhouse conditions (30°C ± 4°C, 16-h photoperiod). For seed multiplication, plants were propagated in the greenhouse and fertilized with 0.5% ammonium sulfate every 2 weeks until flowering.

Cultivation of Rhizobacteria and Pathogens

Bacterial strains used in this study are listed in Table II. *Pseudomonas fluorescens* strain WCS374r and derived mutant strains were grown for 24 to 28 h at 28°C on KB (King et al., 1954) agar plates. Bacterial cells were scraped off the plates and suspended in sterile saline (0.85% NaCl). Densities of the bacterial suspensions were adjusted to the desired concentrations based on their optical density at 620 nm.

Magnaporthe oryzae isolate VT5M1 (Thuan et al., 2006) was grown at 28°C on half-strength oatmeal agar (Difco). Seven-day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light (combination of Philips TLD 18W/08 and Philips TLD 18W/33) for 7 d to induce sporulation. Conidia were harvested according to De Vleesschauwer et al. (2006), and inoculum concentration was adjusted to a final density of 1×10^4 spores mL^{-1} in 0.5% gelatin (type B from bovine skin; Sigma-Aldrich G-6650).

Pathogen Inoculation and Disease Rating

Four-week-old rice seedlings (five-leaf stage) were challenge inoculated with *M. oryzae* isolate VT5M1 as described before (De Vleesschauwer et al., 2006). Six days after inoculation, disease was assessed by counting the number of elliptical to round-shaped lesions with a gray center indicative of sporulation of the fungus and expressed relative to nonbacterized control plants.

Induction Treatments

Induced resistance assays were performed basically as described by De Vleesschauwer et al. (2006). Briefly, plants were grown under greenhouse conditions (30°C ± 4°C, 16-h photoperiod) in commercial potting soil (Structural; Snebbout) that had been autoclaved twice on alternate days for 21 min. Rice seeds first were surface sterilized with 1% sodium hypochlorite for 2 min, rinsed three times with sterile, demineralized water, and incubated on wet sterile filter paper for 5 d at 28°C to germinate. Prior to sowing in perforated plastic trays (23 × 16 × 6 cm), roots of germinated seeds were dipped in bacterial suspensions (5×10^7 cfu mL^{-1}) for 10 min. In addition, the bacterial inoculum was thoroughly mixed with the potting soil to a final density of 5×10^7 cfu g^{-1} and, 12 d later, applied a second time as a soil drench. In control treatments, soil and rice plants were treated with equal volumes of sterilized saline.

For chemical induction of resistance, plants were treated with BTH at 3 d prior to challenge inoculation. BTH (BION 50 WG), formulated as a water-dispersible granule containing 50% active ingredients, was dissolved in sterilized demineralized water for use and applied as a soil drench. Control plants were treated with an equal volume of water. BTH was a kind gift from Syngenta Crop Protection.

Evaluation of Plant Colonization by *P. fluorescens* WCS374r and Mutants

Bacterial colonization of the plant roots was determined by the time the bioassays were discontinued. Roots of three plants of each treatment were rinsed to remove most of the soil, weighed, and macerated in sterile demineralized water. Serial dilutions were plated on KB agar supplemented with the appropriate antibiotics: kanamycin (25 μg mL^{-1}), tetracycline (20 μg mL^{-1}), and rifampicin (200 μg mL^{-1}). Bacterial counts were made after incubation for 24 h at 28°C. Possible spreading of root-inoculated bacteria to distal leaves was checked as stated by De Vleesschauwer et al. (2006).

Purification of Pseudobactin

Bacteria were grown in liquid standard succinate medium (Meyer and Abdallah, 1978), and pseudobactin was extracted and purified according to Meziane et al. (2005). To avoid contamination with SA or pseudomonine, mutant 4B1 was used.

Application of Purified Compounds

For experiments in which purified pseudobactin and/or SA were applied to rice seedlings, plants were grown in a hydroponic gnotobiotic system. Surface-sterilized rice seeds were germinated for 5 d at 28°C on wet filter paper. After incubation, germinated seeds were sown in perforated plastic trays (23 × 16 × 6 cm) filled with sterilized vermiculite and supplemented with half-strength Hoagland solution. Every 3 d, 0.5 L of the nutrient solution was added to each tray containing 12 seedlings. In this model, various concentrations of pseudobactin and SA were applied to the plants at 3 d before challenge by including the desired concentration in Fe-EDTA-free nutrient solution (Acros).

Visualization of Defense Responses

To gain more insight into the cytomolecular mechanisms underlying pseudobactin- and BTH-induced resistance against *M. oryzae*, intact leaf sheath assays were performed as described by Koga et al. (2004). Briefly, leaf sheaths of the sixth leaf of rice plants at the 6.5-leaf stage were peeled off with leaf blades and roots. The leaf sheath was laid horizontally on a support in plastic trays containing wet filter paper, and the hollow space enclosed by the sides of the leaf sheaths above the mid vein was filled with a conidial suspension (5×10^4 conidia mL^{-1}) of *M. oryzae*. Inoculated leaf sheaths were then incubated at 25°C with a 16-h photoperiod. When ready for microscopy, the sheaths were hand trimmed to remove the sides and expose the epidermal layer above the mid vein. Lower mid vein cells were removed to produce sections three to four cell layers thick. For time-course experiments, sheath sections were generally sampled at 18, 24, 30, 36, 48, and 72 hpi, and at least six trimmed sheath tissue sections originating from different plants were used for each sampling time point.

Phenolic compounds were visualized as autofluorescence under blue light epifluorescence (Olympus U-MWB2 GPF filter set; excitation at 450–480 nm, dichroic beamsplitter of 500 nm, barrier filter BA515). To detect H_2O_2 accumulation, staining was according to the protocol of Thordal-Christensen et al. (1997) with minor modifications. Six hours before each time point, trimmed sheath segments were vacuum infiltrated with an aqueous solution of 1 mg mL^{-1} DAB-HCl (pH 3.8) for 30 min. Infiltrated segments were then further incubated at room temperature in the above-mentioned DAB solution until sampling. DAB polymerizes in the presence of H_2O_2 and endogenous peroxidase to form a brownish-red precipitate that can be easily visualized using bright-field microscopy. Specificity of the DAB staining was verified by adding 10 mM ascorbic acid. For protein cross-linking, staining was performed as described by Mellersh et al. (2002). Trimmed sheath segments were submerged in 1% SDS for 24 h at 80°C, stained in 0.1% Coomassie Brilliant Blue in 40% ethanol/10% acetic acid for 15 min, and subsequently rinsed in a solution of 40% ethanol/10% acetic acid. For analysis of callose deposition, trimmed sheaths were stained for 5 min in a solution containing 0.01% (w/v) aniline blue and 0.15 M K_2HPO_4. To visualize cell wall modifications, safranin-O staining was performed according to Lucena et al. (2003) by incubating cut sheath segments in 0.01% safranin-O in 50% ethanol for 2 min. After staining, trimmed sheath segments were mounted in 50% glycerol. Images were acquired digitally (Olympus Color View II camera) and further processed with the Olympus analySIS cell^F software.

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR Analysis

Total RNA was isolated from frozen leaf tissue using the Invisorb Spin Plant RNA Mini kit (Invitek) and subsequently treated with Turbo DNase according to the manufacturer's instructions (Ambion/Applied Biosystems). Before first-strand cDNA synthesis, the absence of genomic DNA was confirmed by PCR. RNA concentration was checked before and after Turbo DNase digestion. First-strand cDNA was synthesized from 2 μg of total RNA using Affinityscript reverse transcriptase and oligo(dT) primers (Stratagene/Bio-Connect), according to the manufacturer's instructions. The following primer sequences were used: for *actin* (Os03g50890; similar to AB047313.1), forward 5′-GCGTGGACAAAGTTTTCAACCG-3′ and reverse 5′-TCTGGTACCCTCATCAGGCATC-3′; for *PBZ1* (Os12g36880; similar to D38170), forward 5′-CCCTGCCGAATACGCCTAA-3′ and reverse 5′-CTCAAACGCCACGAGAATTTG-3′; and for *PR1b* (Os01g28450; similar to U89895), forward 5′-GGCAACTTCGTCGGACAGA-3′ and reverse 5′-CCGTGGACCTGTTTACATTTT-3′. For each primer pair, the optimal annealing temperatures were predetermined by gradient PCR using a Thermocycler (Bio-Rad). Furthermore, for each target, primer concentrations were optimized by performing a primer titration. Quantitative PCR amplifications were conducted on optical 96-well plates with the Mx3005P real-time PCR detection system (Stratagene), using Sybr Green Master Mix (Stratagene/Bio-Connect) to monitor double-stranded DNA synthesis. The expression of each gene was assayed in triplicate in a total volume of 25 μL including a passive reference dye (ROX) according to the manufacturer's instructions (Stratagene). The thermal profile used consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 60 s. Fluorescence data were collected during the annealing stage of amplification. To verify amplification of one specific target cDNA, a melting-curve analysis was included according to the thermal profile suggested by the manufacturer. The amount of plant RNA in each sample was normalized using actin (Os03g50890) as an internal control, and samples collected from control plants at 0 hpi were selected as a calibrator. The generated data were analyzed with the Mx3005P software (Stratagene). For all amplification plots, the optimal baseline range and threshold cycle values were calculated using the Mx3005P algorithm. Gene expression in control, BTH-treated, and pseudobactin-treated samples was expressed relative to the calibrator and as a ratio to actin expression using the measured efficiency for each gene.

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